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# 3-Amino-2-Piperidinequinoline A Novel Natural Product-Inspiried Synthetic Compound with Antimalarial Activity

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# 3-AMINO-2-PIPERIDINEQUINOLINE A NOVEL NATURAL PRODUCT-INSPIRED SYNTHETIC COMPOUND WITH ANTIMALARIAL ACTIVITY

by

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# ABSTRACT

Malaria afflicts about 500 million people worldwide thus causing significant global economic toll. The drugs available to treat the disease are rapidly losing their efficacy because of widespread prevalence of drug resistant parasites. Thus there is an urgent need to discover novel malaria therapeutics. This research is focused on to study the properties of a novel natural-like synthetic scaffold and analyze its selectivity, and cellular mechanism of action in *Plasmodium falciparum.* We have identified a novel compound, 3-amino-2-piperidinequinoline (APQ), which we termed UCF401. APQ demonstrated $IC_{50}$ at submicromolar concentrations against *Plasmodium falciparum* using the SYBR Green-I fluorescence assay measuring cellular proliferation. This compound also demonstrated low cytotoxicity against the NIH3T3 and HEPG2 cells using MTS assays, showing an $IC_{50}$ of 174 μM and 125 μM respectively, suggesting of excellent selectivity. We evaluated the compliance of APQ with Lipinski's parameters and determined the *in vitro* physicochemical profiles of the compound. Our results show that APQ is a Lipinski parameter compliant and has good physicochemical properties. The cellular mechanism of action of APQ was characterized through the assessment of the effects of the compound at different stages of the parasite's intraerythrocytic life cycle. This assay was done by treating a synchronized cell line with the compound at 5X the $IC_{50}$ value and then imaging the cells at 12-hour intervals. We found that APQ arrests parasite development at the trophozoite stage. In addition we determined that APQ is parasitocidal after a 96 h exposure. These results demonstrate that APQ can be considered as a validated hit and/or early lead.

# DEDICATION

To God, for providing me the energy, determination and perseverance,
To my family, for providing me their unconditional support and endless opportunities,
To my dearest friend, Laura Hoyos, for providing the encouragement,
To the DC lab team for providing the perfect environment,
And to myself… for combining all of this together.

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I would like to thank my Principal Investigator and mentor Dr. Debopam Chakrabarti for demanding nothing but excellence, Ph.D. Candidate Bracken F. Roberts for helping me achieve such excellence, Dr. Kenneth Teter for his invaluable feedback and direction throughout this endeavor, and finally the Drug Lab team for their vital input and support.

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# CHAPTER ONE: INTRODUCTION

## Malaria Causing Agent

Malaria is recorded by mankind throughout history, going back more than 4,000 years. It was often referred to as the "tertian" or "quartan" fever, due to its characteristic symptoms and patterns of expression [1]. The existence of these symptoms has been recorded by almost every major ancient civilization and its virulence and mortality have influenced many historical outcomes [1]. The term Malaria comes from the Italian words mal-aria, meaning "bad air" [1]. It is not know who exactly coined the term but the disease's causing agent was discovered by French surgeon Alphonse C. Laverans [1]. This pathogen is identified as a single cell eukaryotic microorganism belonging to the phylum Apicomplexa, (due to the presence of an organelle called the apicoplast), and the genus called Plasmodium, named by Ettore Marchiafava in the early 1900's [2]. There are several Plasmodium species known to infect humans and cause disease, such as *P. falciparum, P. vivax, P. ovale, P. knowlesi, and P. malariae*; which are distributed worldwide, mostly in tropical and subtropical areas often having overlap within different species. Out of these species *P. falciparum* causes the most severe form of the disease and it is responsible for approximately a million deaths per year. *P. vivax* as well as *P. ovale* have dormant life stages (hypnozoites), they harbor in the hepatocytes and emerge when conditions allow. *P. malariae* uses a three-day life cycle (quartan cycle), while all the other species use a two-day life cycle (tertian cycle). This species can also cause a chronic, long lasting infection and disease. Finally, *P. knowlesi* is known to be able to infect both humans and monkeys [1].

**Plasmodium Life Cycle**

The life cycle of the malaria pathogen is very complex and it involves the use of two hosts (Figure 1). The first host is the *Anopheles* female mosquito. When pregnant, the egg-carrying mosquito must have a blood meal in order to sustain the nutritional requirements of the eggs. Once the *Anopheles* mosquito bites a malaria-infected individual, the mosquito uptakes the infected blood. Some of the parasites uptaken in this meal are in the gametocyte sexual life-stage, and they will continue to develop as they travel, fusing with the lumen of the mosquito's gut, transforming into a sporozoite-releasing oocyst. These sporozoites then travel to the salivary glands of the mosquito and stay there, waiting to be spread onto the next host when the mosquito feeds again and releases infected saliva into the host's bloodstream [1]. This cycle is called the Sporogonic Cycle, and it usually takes from 10-18 days, depending on the conditions and species of Plasmodium [3]. The next cycle is called the Exo-erythrocytic cycle, which starts when the newly injected sporozoite travels through the bloodstream until reaching the liver, binding to and invading hepatocytes. Over the next 5-25 days these sporozoites reproduce exponentially through asexual binary fission, forming a compartmented structure called the liver schizont and reaching populations of up to 30,000 individual sporozoites, eventually rupturing the schizont and the hepatocyte, releasing merozoites. These merozoites escape the liver and travel through the blood stream seeking for the next target, the red blood cell (RBC), entering into the Human Blood Stage or the Erythrocytic life cycle. At this stage the merozoites invade the RBC and continue to mature, turning into immature trophozoites (ring stage), where then the cycle splits into some parasites entering the sexual erythrocytic cycle while others enter into the asexual erythrocytic cycle, maturing into trophozoites, followed by schizonts until finally rupturing the RBC and

escaping as merozoites, continuing the erythrocytic cycle. In the sexual erythrocytic cycle the immature trophozoites develop into male and female gametocytes, which are then uptaken by the *Anopheles* mosquito upon feeding, restarting the entire cycle again [4]. This complex life cycle makes it very difficult for researchers to find specific targets for the development of therapeutic treatments to combat this disease.

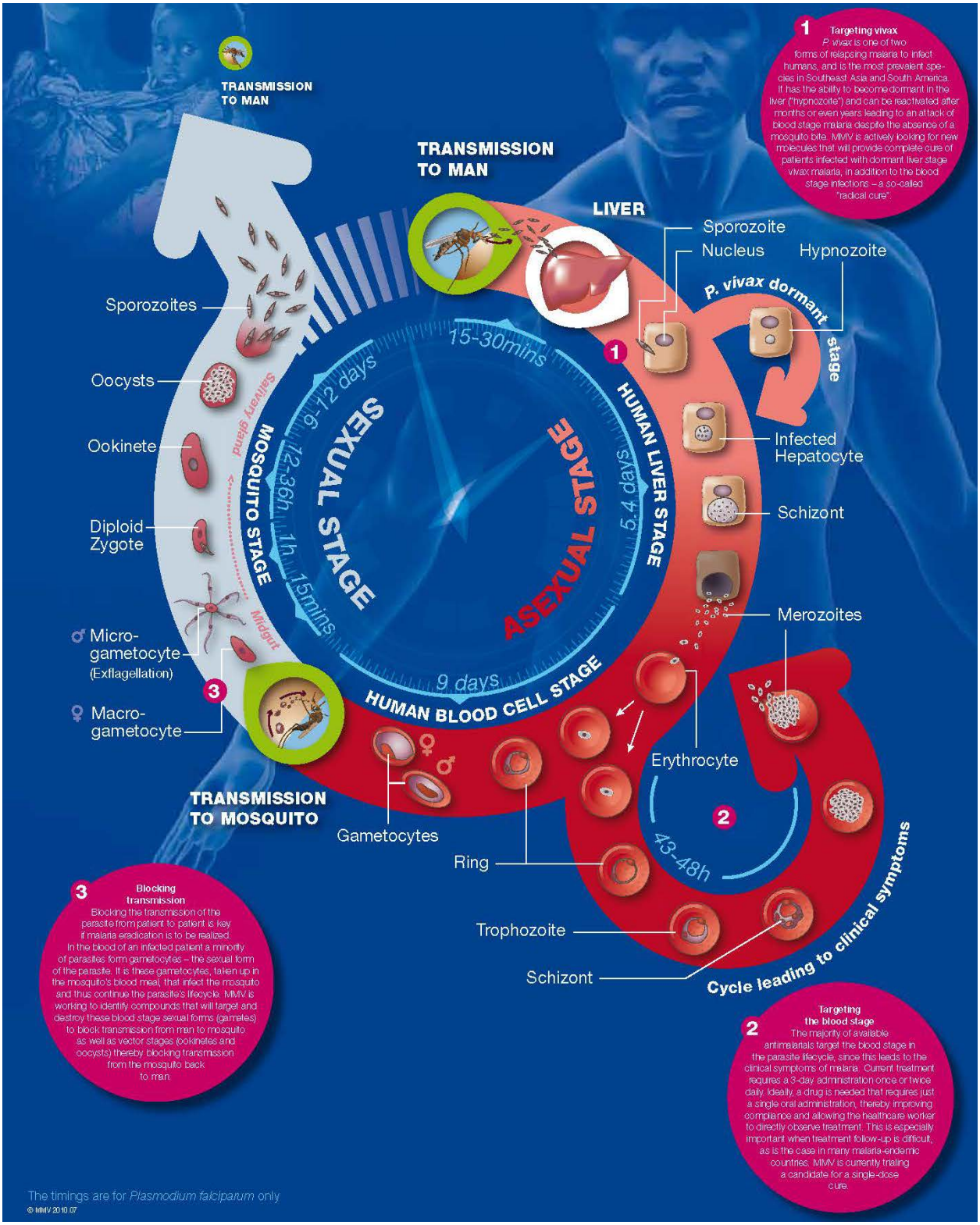



**Figure 1: Plasmodium falciparum spp. life cycle [5]**

## Disease and Symptoms

The Malaria disease clinical symptoms start developing once the exponentially reproduced merozoites escape from the hepatocytes into the bloodstream, binding, invading and rupturing the RBC, inhibiting it from performing its function, and expelling hemozoin into the bloodstream. Hemozoin is a toxic byproduct of the digestion of hemoglobin, which initiates a strong cytokine response [6], resulting in common symptoms such as fever, chills, sweats, headaches, nausea, vomiting, and general body aches. All of these symptoms are related to a typical inflammatory response. Infected RBCs also become more adhesive towards the endothelial tissues of the capillaries, obstructing proper blood flow to crucial organs. If untreated, malaria can complicate and cause severe symptoms related to organ failure and sepsis, such as cerebral malaria, coagulation abnormalities, hemoglobinuria, severe anemia due to hemolysis, acute respiratory distress syndrome (ARDS) caused by the decrease of oxygen delivery capacity, and enlarged liver and spleen [1].

## Preventative Measures

Since malaria is transmitted through a vector (*Anopheles* female mosquito), reducing the incidence of bites and the overall population of this mosquito species is the current approach for the prevention of malaria bouts. This is called vector control and is done by applying simple solutions that have a much greater impact; taking into consideration that the populations mostly affected by this disease are critically underdeveloped nations that lack the resources necessary to efficiently combat the problem at hand. Thus, the preventative measures must be simple to follow and affordable to maintain.

The most prevalent preventative measures being taken today are:

- The use of Insecticide-treated bed nets and curtains (ITBC), which have proven to be highly effective at reducing the morbidity and mortality of malaria in African countries, since mosquitoes often feed at dusk and/or night [7], reducing the mosquito bites, reduces the cases and the spread of malaria. The setback with the implementation of this measure is affordability by the individual or community and non-compliance. To bypass these setbacks, communities depend on donors and the necessary education to increase the level of compliance.
- The use of indoor residual spraying (IRS) involves the use of insecticides to reduce the population of the *Anopheles* mosquito. These insecticides vary from organochlorines, pyrethroids, organophosphates and carbamates. They are all highly effective, and their use depends mostly on susceptibility of the *Anopheles* species, environmental and human safety and cost-effectiveness. DDT, an organochlorine, is the most widely used insecticide, but its use is restricted for public health reasons, and to prevent the spread of resistance displayed by the *Anopheles* mosquito species [8]. The major setback to this measure is the affordability of the insecticide products, the environmental consequences, and the development of resistance to the chemical compounds by the Anopheles species. Again, the efficacy of this preventative measures depends on donors, global funding, and on education of the prudent use of insecticides to prevent resistance development [8].

**Current Treatments**

In addition to the preventive measures, a chemotherapeutic approach is also necessary in order to prevent disease, if used prophylactically, or to cure disease when a case occurs. These

measures alone do not prevent malaria emergence efficiently but do play a direct role in the decrease of mortality from an infection and the decrease of disease-spread. Thus, as mentioned above, it is critical to implement a combination of preventive and therapeutic measure to control the disease as efficiently as possible.

There are many chemotherapeutic agents that have been found throughout history thanks to scientific research and drug development technologies. However, these compounds are also victims of affordability, sustainability, non-compliance, adverse health effects, and fast and widespread pathogenic resistance development. Hence, it is critical that the efforts and the search for effective antimalarial compounds must be continued globally to support drug development research and drug mass-distribution for the hardest-hit nations. In addition to patient and communal education, which should place focus on patient compliance.

The most commonly used chemotherapeutic agents belong to several families of compounds, which are:

- Endoperoxides
- 4-aminoquinolines
- 8-aminoquinolines
- Antifolates
- Sulfonamides
- Antibiotics
- Amino alcohols
- Others

The major categories of chemotherapeutic agents and their mechanism of action will be discussed below (Figures 2 and 3).

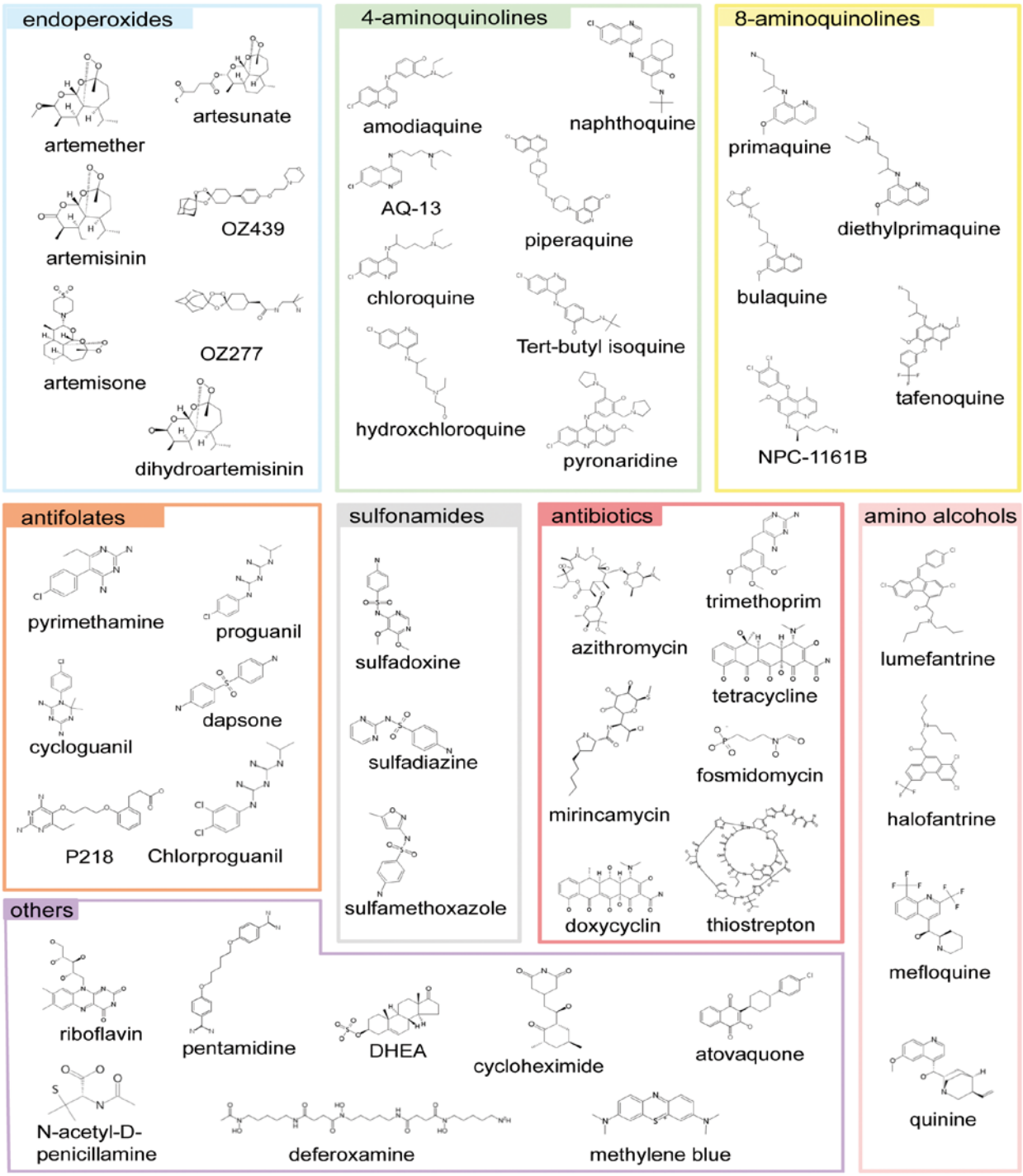



**Figure 2: Families of Chemotherapeutic Antimalarial Compounds [9].**

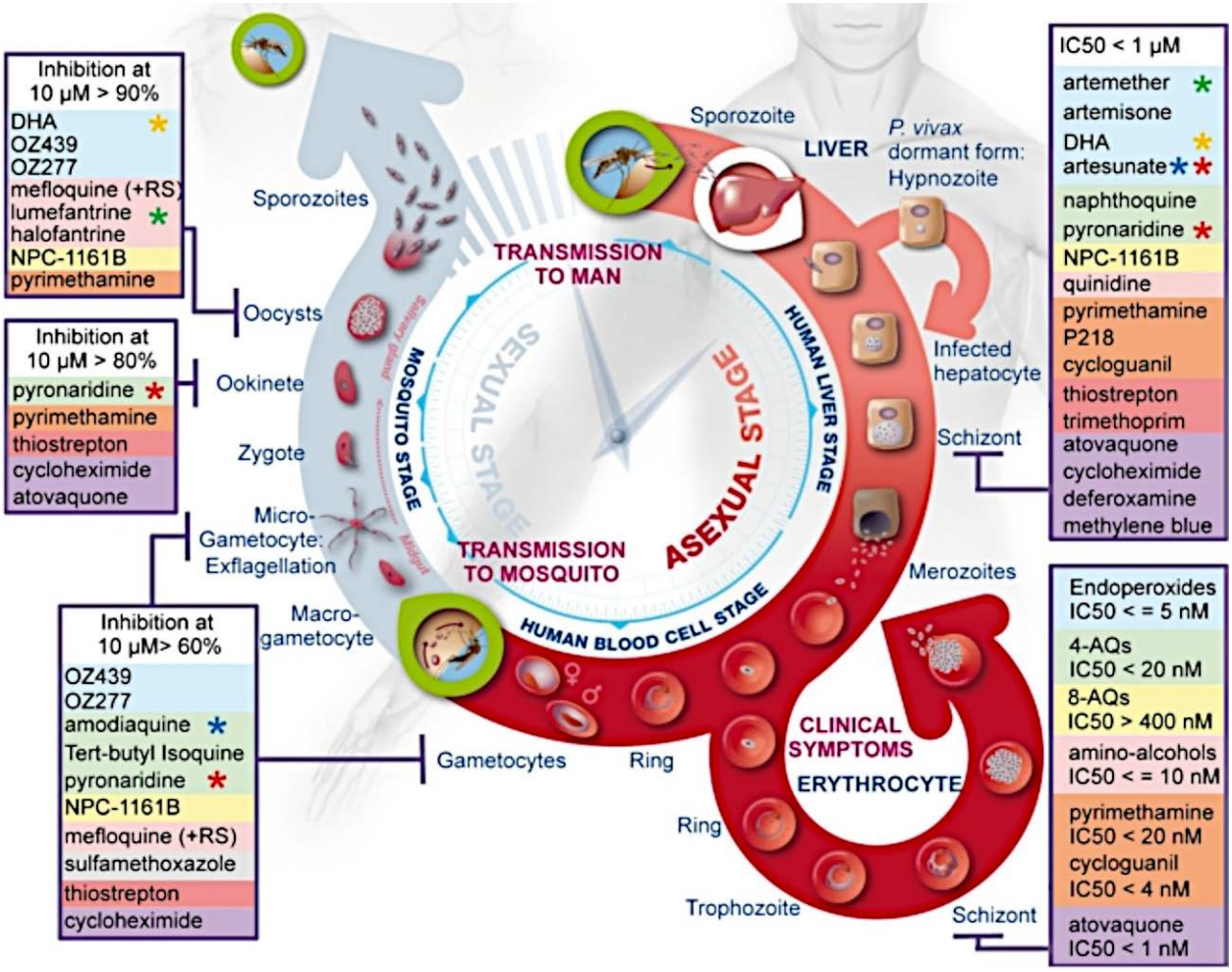



**Figure 3: The different plasmodial life stages at which the most commonly used antimalarial compounds inhibit proliferation [9].**

4- and 8- Aminoquinolines:

The most important compound pertaining to this family of agents is chloroquine. Chloroquine has the ability to be used as both a prophylactic agent and a therapeutic agent as well. Chloroquine destroys the parasite by entering into the parasite digestive vacuole and binding hematin, which is a toxic (to the parasite) by-product of hemoglobin digestion by the parasite. Normally, the hematin is polymerized into hemozoin crystals to reduce its toxicity to the parasite. This detoxification is disrupted by chloroquine and the build-up of toxic hematin

destroys the parasite [10]. However the widespread use of chloroquine has rendered it almost useless throughout Africa due to the widespread development of drug resistance [1].

Antifolates and Sulfonamides:

Antifolates are chemical compounds that disrupt the folate biosynthesis pathway of Plasmodium species; this pathway is essential for DNA and RNA synthesis and the metabolism of certain amino acids. Amongst the antimalarial drugs currently in clinical use, the antifolates have the best-defined molecular targets, namely the enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) [11]. The most commonly used antifolate is Pyrimethamine. However, its use is limited by the ability of the parasite to uptake biosynthesized folates from the environment, where it is widely available as folic acid, defeating the purpose of an antifolate. Nevertheless, if used in combination with a sulfonamide such as sulfadoxine, the synergistic effect between the two compounds inhibits the parasite from uptaking exogenous folic acid [12]. This combination is widely used to substitute chloroquine usage, where resistance development has limited its effectiveness.

Endoperoxidases:

The most important and effective compound of this family, and in fact the most potent antimalarial compound used today is artemisinin. These compounds, derived from the leaves of the Artemisia annua plant, are extremely effective, providing a broad-stage inhibitory capacity and a very fast response compared to other antimalarial agents. Artemisinin is also effective against multi-drug resistant parasites. However, when artemisinin derivatives are administered as a monotherapy, the fast half-life and rapid excretion requires a prolonged treatment (minimum

duration, 7 days) to achieve a cure. On top of this, its fast-acting capacity and the need for prolonged administration, mixed with the inevitable patient non-compliance, makes this treatment vulnerable to developing resistance. For this reason, artemisinin is usually given in combination with a drug from the same family that has a longer half-life; this strategy is known as artemisinin-based combination therapy (ACT). The reason for ACTs success is that the short-acting but highly effective artemisinin delivers a rapid reduction in parasitemia, with the remaining parasites being removed by the less effective but more slowly eliminated analogs. The ACTs have the additional benefit of dramatically reducing the production of gametocytes, the sexual stage of the parasite as a consequence of the rapid reduction in the parasite biomass. Displaying activity against both, the more mature asexual-stage parasites (the precursors of the sexual stages), as well as gametocytocidal activity against early gametocytes (stage I–III) [13].

Antifolates and Sulfonamides, 4- and 8- Aminoquinolines and Endoperoxidases are the most widely used drug compounds against the war on malaria. The other chemical families of antimalarials and their compounds are often used in rare cases where resistance is emerging towards the mainstream drugs, or they can also be given in combination with these commonly used antimalarials. Shown below, in Table 1, is a summary of the most widely used antimalarial compounds, the region of most use, and the level of resistance acquired against it. This summary sheds light onto the global need for further scientific research with the goals of optimizing already present antimalarial compounds and creating and testing analogs of these already known compounds.

| Common name | Chemical class | Clinical use | Resistance |
|---|---|---|---|
| Artemisinins (artemether, artesunate, dihydroartemisinin) | Sesquiterpene lactone endoperoxide | In artemisinin-based combination therapies (ACTs) | Possibly emerging |
| Lumefantrine | Arylamino alcohol | Most common first-line antimalarial therapy in Africa, in combination with artemether | No evidence of high-level resistance |
| Amodiaquine | 4-Aminoquinoline | In combination with artesunate in parts of Africa | Limited cross-resistance with chloroquine |
| Piperaquine | Bisquinoline | In combination with dihydroartemisinin in parts of southeast Asia | Observed in China following single-drug therapy |
| Mefloquine | 4-Methanolquinoline | In combination with artesunate in parts of southeast Asia | Prevalent in southeast Asia |
| Pyronaridine | Acridine-type Mannich base | Being registered for combined use with artesunate | No cross-resistance with other drugs reported |
| Quinine/quinidine | 4-Methanolquinoline | Mainly for treating severe malaria, often with antibiotics | Exists at a low level |
| Atovaquone | Naphthoquinone | In combination with proguanil (a biguanide) for treatment or prevention | Has been observed clinically |
| Chloroquine | 4-Aminoquinoline | Former first-line treatment for uncomplicated malaria | Widespread |
| Pyrimethamine | Diaminopyrimidine | For intermittent preventive treatment, combined with sulphadoxine (a sulphonamide) | Widespread |
| Primaquine | 8-Aminoquinoline | For eliminating liver-stage parasites, including dormant forms of *Plasmodium vivax* | Unknown |

**Table 1: Antimalarial compounds. Their chemical class, clinical use and level of resistance [14].**

The best way to treat malaria is through a thorough and effective combination of preventative measures, to reduce the overall cases of infection, and an aggressive and compliant chemotherapy using the available drugs developed to treat and cure present occurrences.

**Significance**

The malaria disease poses a great problem for humanity because it afflicts 40% of the global population, up to 3.3 billion people that live in areas in danger of malarial transmission. These areas comprise up to 106 countries, all of which are considered 3rd world countries from a socioeconomic perspective. This poses an even greater issue due to their lack of resources and their dependency on external aid from developed nations and organizations such as the World Health Organization (WHO). According to the WHO, an estimated 219 million cases of malaria with 660,000 deaths occurred in the year 2010, Up to 90% of deaths occur in the African region and 86% of these deaths occur in children under the age of 5. The great incidence of malaria throughout history makes it the highest selective pressure placed on humanity, killing more humans than any other disease. But there is hope for better statistics in the future, thanks to the conjunction of global awareness and efforts as well as scientific research and their developments; malaria mortality rates have decreased by more than 25% globally since 2000 [15]. If these global efforts continue to flourish into solutions for the issue at hand, then this trend is going to continue on to eradicate malaria from humanity. There are many obstacles to accomplish this, such as the ability of the parasite to develop drug resistance due to its complex genome, fast rate of reproduction, and its complex life cycle. These issues make it difficult for drug developers to find successful molecular targets. Nevertheless, malaria has been eradicated in developed nations by taking the necessary measures and one can argue that it will be accomplished globally if the continued global economic support and successful scientific findings for alternative and efficient treatments persists.

**Purpose**

The drugs available to treat Malaria are rapidly losing their efficacy because of widespread prevalence of drug resistant parasites. Thus there is an urgent need to discover novel malaria therapeutics. This research is focused on the study of the properties of a novel natural-like synthetic scaffold and the analysis of its selectivity, and cellular mechanism of action in *Plasmodium falciparum.* We aim to show its strength as a validated hit in order to promote it to early lead stage, in accordance to the parameters set by the Medicines for Malaria Venture (MMV).

# CHAPTER TWO: THE POTENTIAL OF NATURE-LIKE PRODUCTS IN ANTIMALARIAL DRUG DISCOVERY

## Nature-like Products

Historically, natural products (NPs) have been the central dogma of drug discovery [16]. From the use of plant extracts to treat disease to the discovery of antibiotics to treat infections (bacterial, fungal or parasitic), natural scaffolds have been the main resource for drug research and development [16]. The vast biodiversity of organisms that throughout millions of years of evolution have developed the necessary chemical arsenal to interact with their specific environment and ensure their survival and fitness is the main origin for the myriad of natural-product derived drugs in current use and the incalculable amount of possibilities yet to be found [16]. Natural products are isolated from plants, algae, insects, bacteria, yeast, and animals. Approximately 25% of drugs in use today are derived from natural products, and in certain therapeutic areas, the impact is even higher, e.g., anti-cancer (74%) and anti-bacterial (78%) [17]. Thus, it is evident that these chemical templates produced by nature will yield higher quality and more accurate results in the drug research field, since these were originally synthesized to interact with biologically active molecules, and produced to outcompete other life forms [16].

Natural products have proven to be effective drugs. However, in the drug discovery and research field the majority of these products tend to be good lead compounds but often do not meet the demands for druggability. Druggability are the physicochemical, biochemical, pharmacokinetic and safety properties of drugs [18]. Modification or optimization of these

products, such as the elimination or substitution of functional groups or chirality centers, is often necessary in order to mitigate the undesired properties and activities of the lead NP compound while retaining the desired biological activity [18]. This leads to the synthesis of novel compounds that maintain a natural chemical template or scaffold and the enhancement of druggability and biological activity on desired targets as well as the decrease of chemotherapeutic instability and toxicity for the host, through the use of structure-activity relationship analysis and medicinal chemistry [18]. These novel compounds synthesized using natural scaffolds are known as synthetics derived natural products (SNPs) or nature-like products.

**Structure Activity Relationship**

The structure-activity relationship (SAR) analysis allows for the interchangeability of the chemical structure and configuration of a biologically active molecule in order to enhance its potency and effectiveness against its target and optimize its physicochemical properties and metabolic stability. This is done through the use of medicinal chemistry and the creation of chemical analogs of a lead compound in order to comply with the druggability parameters and standards of drug development and maintain the chemotherapeutic effect desired [19].

**Pharmacokinetic and Physicochemical Parameters**

Since Malaria affects mostly areas of economic turmoil, an efficient drug ideally needs to be administered by the oral route, in order to eliminate the need of licensed professionals administering the drug and the use of resources that are already scarce in these areas e.g. syringes.

In General, a drug must comply with the following:

- Must bind tightly to the biological target *in vitro* and *in vivo*
- Must be permeable to one or more physiological barriers such as cell membranes
- Bioavailability must be sustained for drug to take proper effect
- Must be excreted from host by metabolism

For a chemotherapeutic to have an effective physicochemical profile, it must comply with Lipinski's rule of five (RO5) criteria [20]. Which are:

- Molecular weight of < 500 g/mol
- cLogP < 5
- Less than 5 H-bond Donors (OH and NH groups)
- Less than 10 H-bond Acceptors (N and O groups)
- Polar surface area of less than 140 Angstroms squared [21]

The chemotherapeutic also must have drug-like properties and a good ADME (absorption, distribution, metabolism, excretion) profile for oral bioavailability. These properties can be extrapolated from the elucidation of the following pharmacokinetic values:

- Metabolic Stability:

  Measures the stability of the compound in the body and hence the excretion rate by metabolism. Poor metabolic stability means that compounds pass through liver metabolism readily after intestinal absorption via the portal vein, negatively affecting the bioavailability and increasing rapid elimination from the body. Liver

metabolism often alters the chemical structure of the compound, rendering it ineffective. Microsomal stability assays are done to measure the disappearance rate of the molecule in the presence of hepatic enzymes, such as cytochrome P450 (CYP450) and Uridine 5'-diphospho-glucuronosyltransferase (UGT) [22], which are standard enzymes used in drug development research. This value is measured in percentage of drug available after 60 minutes [23].

- Solubility:

  Solubility of the compound plays a central role in absorption through biological membranes. Poor solubility correlates with poor absorption. As a rule of thumb, a compound with an average potency of 1mg/kg should have a solubility of at least 0.1g/L at pH 7.4 to be adequately soluble. If a compound with the same potency has a solubility of less than 0.01g/L it can be considered poorly soluble [24]. Solubility values are established through kinetic or thermodynamic solubility assays.

- Distribution:

  After a drug enters the systemic circulation, it is distributed to the body's tissues. Distribution is generally uneven because of differences in blood perfusion, tissue binding (eg, because of lipid content), regional pH, and permeability of cell membranes [25]. Lipophilicity is a measure of several distribution factors and the molecules behavior in plasma [26]. It also shows relationship to plasma protein binding, permeation and solubility [26]. Lipophilicity values are found through partition coefficients clogP for neutral compounds, using a two-phase system of

octanol and water, and clogD for ionizable compounds using a pH range, usually logD at pH 7.4 [23]. A good partition coefficient value is less than 5.

- Permeability

  As stated above, efficient antimalarials are ideally designed to be administered via oral route and absorbed through the intestinal lumen, the chemotherapeutics must be able to permeate through the several membranes to reach the systemic circulation. The most used approaches to study the permeation of the molecule across these barriers are enterocyte models such as Caco-2, or even an artificial membrane-assay called parallel artificial membrane permeability assay (PAMPA) [23][27].

- Plasma-protein binding:

  The extent of binding to plasma influences the way in which a drug distributes into tissues in the body. If a compound is highly bound, then it is retained in the plasma, which results in a low volume of distribution. This may impact on the therapeutic effects of the compound by limiting the amount of free compound available to act on the target molecule. Extensive plasma protein binding also limits the amount of free compound available to be metabolized reducing the clearance of the compound [28]. Degree of binding is most usually elucidated using methods based on rapid equilbrium dialysis (RED). Results are usually expressed as percentage bound after 4 h using two varying concentrations, usually 1 μM and 10 μM [29].

**Medicines for Malaria Venture (MMV) and their compound progression criteria**

MMV, established in 1999 in Geneva, was the first public–private partnership of its kind to tackle a major global disease. This not-for-profit organization brings together public, private and philanthropic partners to fund and manage the discovery, development and delivery of affordable new medicines for the treatment and prevention of malaria in disease-endemic countries. They have established the standards and criteria to follow in the research and development of new chemotherapeutics against malaria in all of the pre-clinical stages of drug developments. The parameters for the two earliest stages of drug development (primary hits and validated hits) are as follows:

1. Screening Hits
    a. $IC_{50}$ at <1 μM in P. falciparum strains 3D7, HB3, Dd2, NF54, and W2
2. Validated Hits
    a. Reproducibility of primary results ($IC_{50}$ at <1 μM)
    b. If natural products are used, they must be acquired from a guaranteed source and their active species must be known.
    c. Activity against mammalian whole cells (minimum of two, being HepG2 one of them) shows >10-fold selectivity
    d. Has drug-like properties and does not violate Lipinski's RO5.
    e. Computational ADME satisfactory
    f. Some basic SAR apparent
    g. Biological effects established: any stage specificity of action against blood stages (rings, trophozoites, or schizonts); cidal or static action determined.

Once compliant with these parameters, the researcher can move on to the next pre-clinical drug development stages which are early leads and late leads, before the drug can be submitted as a development candidate [30].

**3-amino-2-piperidinequinoline**

Preliminary research in our lab has focused in screening for antimalarial activity and selectivity of various natural product-like compounds commercially acquired from Asinex, a renowned chemical library provider. These compounds, derived from natural scaffolds, are enriched with features of known antimalarials, such as fused aromatic rings and protonable nitrogens, but excluding peroxide bridges, which are the leading characteristic of artemisinins. Within these compounds, primary hits with submicromolar antimalarial activity and broad selectivity were identified; 3-amino-2-piperidinequinoline (APQ), which we termed UCF401 is one of these primary hits.

APQ is characterized for having a quinoline scaffold with an amino group, which resembles widely used and proven antimalarials, this scaffold is also enriched with a piperidine group and a fluoride substituent. The chemical structure of APQ is advantageous for drug development since it does not posses any chiral centers, which translates into a single isomer structure without the need to worry for the stereoselectivity of the molecular target [31].

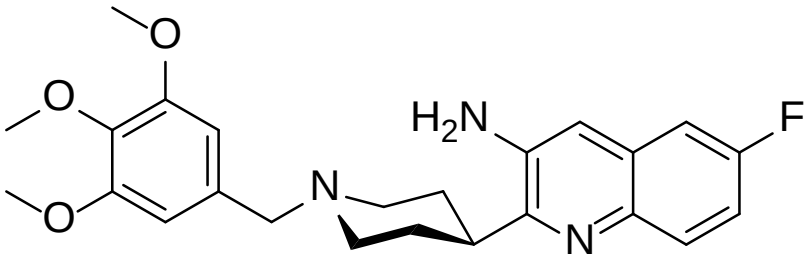



**Figure 4: 3-amino-2-piperidine-quinoline (APQ) chemical structure**

As a primary hit, APQ will be tested and analyzed in compliance with the MMV screening and validated hit parameters in order to promote the compound to early lead stage if the compound proves to be successful in all these criteria. We will be showing antimalarial potency, cytotoxicity assays in two human cell lines, cellular mechanism of action, and cidal vs. static activity for optimum dosage exposure time. The results of the antimalarial activity and selectivity will be validated through Z-scores and the assays will be independently repeated three times for reproducibility and further validation. *In vitro* Lipinski and ADME profiles for APQ were preliminarily acquired from the Sanford Burnham Pharmacology Core Facility at Lake Nona, Orlando. Finally, structure-activity relationship analysis will be performed on seven APQ analogs commercially acquired from the Asinex APQ library. These analogs will be tested for antimalarial efficacy and cytotoxicity in human cell lines. The results of this research are presented below.

# CHAPTER THREE: METHODOLOGY

## Culturing P. *falciparum*

*P. falciparum* Dd2 and 3D7 strains will be cultured using a modified Trager and Jensen method [32] in RPMI media with L-glutamine (Invitrogen) and supplemented with 25mM HEPES, 26 mM $NaHCO_3$, 2% dextrose, 15mg/L hypoxanthine, 25mg/L gentamycin, and 0.5% Albumax I. Culture media will be changed daily and incubated at 37°C in 5% $CO_2$ and 95% air.

The 3D7 strain is a chloroquine sensitive strain characterized as chloroquine sensitive, pyrimethamine sensitive, mefloquine sensitive, and Artemisisin sensitive. The Dd2 strain was used as a chloroquine resistant strain with resistance to chloroquine, pyrimethamine, and mefloquine but sensitivity to artemisinin. A blood smear will be taken daily and stained with Giemsa stain to determine parasitemia. When parasitemia reaches above 10-15% the culture will be split down and resupplied with washed and 50% diluted A+ blood, which will be acquired from the Florida Blood Bank.

## Drug Sensitivity Assay and Malaria SYBR Green-I Fluorescence Assay (MSF)

Given that the MSF assay is much cheaper and produces a quality equal to other well established but more complex and expensive methods for detecting *P. falciparum* inhibition, it will be chosen as the preferred method for the purpose of this research [33]. Because the *P. falciparum* host is the RBC, which has no DNA, and since SYBR Green is a known DNA intercalating agent, which will emit excitation-induced light only after binding to DNA. We can use this assay to quantify DNA levels and therefore, to quantify inhibition levels. Lower fluorescence values, means lower DNA concentrations and thus, lower parasitemia.

Different dilutions of the chemical compound in 1 µl of the culture medium will be added to 99 µl of *P. falciparum* culture at a 1% parasitemia and 2% hematocrit in 96-well plates. Maximum DMSO concentration in the culture will not exceed 0.125%. Chloroquine at 1 µM will be used as positive control to determine the baseline value of possible inhibition. Following 72 h incubation at 37ºC, the plates will be frozen at -80ºC. After thawing the plates at room temperature, 100 µl of lysis buffer (containing 20mM Tris-HCL, 0.08% Saponin, 5mM EDTA, 0.8%Triton X-100, and 0.01% SYBR Green I) will be added to each well. Plates will be incubated in the dark for 30 minutes at 37ºC [33], followed by fluorescence emission reading which will be done using a Synergy H4 hybrid multimode plate reader (Biotek) set at 485 nm excitation and 530 nm emission.

**Sorbitol Synchronization of *P. falciparum***

The pRBCs at a stage not later than ring stage and no later than 10-12 h post invasion will be treated with sorbitol by spinning down the culture at 600x gravity to a pellet, 4 ml of 5% sorbitol in $dH_2O$ will be added and the resulting mix will be incubated for 10 min at room temperature. This solution will be centrifuged again at 600x gravity and washed 3 times in malaria culture medium, diluting it to 4% hematocrit. This procedure will be repeated after one cycle (~48 h) and at least once a week to maintain synchronization [34].

**Time Point Assays for Cellular Mechanism of Action Elucidation:**

In order to assess the effects of the chosen chemical compounds at each specific intraerythrocytic life stage of the parasite, a time point analysis will be performed. This will allow further understanding on the cellular mechanism of action of the specific compound.

Elucidating the specific intraerythrocytic stage at which the compound is most effective, while giving some insight as to which molecular target the compound is acting upon. This assay will be done by synchronizing parasitized RBCs (pRBCs) through the sorbitol synchronization protocol and then treating the synchronized cell line with the compound at 5x the $IC_{50}$ value, previously obtained. Parasites will be cultured in the presence and absence of each compound at 5% parasitemia and 1% hematocrit, and incubated under the conditions described above. Treatments will be maintained for 36 h and the morphology of treated parasites will be compared to the control group every 12 h by examining Giemsa-stained thin blood smears.

**Assessment of the effect of parasitocidal versus static activities**

In 24-well plates, 1-mL cultures P. falciparum at 0.5% asynchronous parasitemia and 1.0% hematocrit were treated with 1.5 and 15 µM of APQ, 3 and 30 times the $IC_{50}$ for this compound, respectively. Cultures lacking drug were used as controls. Separate cultures were set up for 24, 48, 72, 96, and 168 h drug exposure, at which time infected RBCs were washed three times and resuspended in fresh RPMI without compound. To monitor for growth after cultures had potentially overgrown, replicate cultures at 72 h were diluted 1:10 into fresh RPMI with or without drug at 1% hematocrit for the 96 and 168 h time points. Media was replaced on alternate days, and fresh erythrocytes at 1% hematocrit were added on opposite days of media replacement. P. *falciparum* growth was followed by microscopy [35].

**Human and Rodent Cell Lines Cytotoxicity Assay**

Compounds were evaluated for cytotoxicity using NIH/3T3 rodent fibroblast cells and HepG2 (human hepatocytes) cells. A 384 well plate was seeded with 2,500 cells/well (total

volume 50µL) and incubated for 24 hours. Serial dilutions of the compound were added to the plate and plates were incubated for an additional 48 hours. 20 µL MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), CellTiter 96® Aqueous non-radioactive cell proliferation assay, Promega] reagent was added to each well and the plates were incubated for an additional 3 hours. Cell viability was obtained by measuring the absorbance at 490nm using Synergy H4 hybrid multimode plate reader (Biotek) [36].

**Sensitivity and Accuracy Statistical Analysis Formula**

In order to validate, recognize, and quantify the sensitivity and accuracy of the assays performed the Z-factor analysis will be utilized. This assay was originally developed, for high throughput screening (HTS) validation [37]. The equation is listed below as Equation 1-1. The Z-factor is a number, which should range from 0 to 1. The closer the score is to the value of 1, the higher the confidence in the results and the higher the accuracy of the values. A value below 0.5 is considered invalid and will require alterations or the assay to be redone. A value between 0.5 and 1 is considered acceptable and valid quality data.

$$Z - factor = 1 \pm 3\left(\frac{(\sigma p - \sigma n)}{(\mu p\ - \mu n)}\right)$$

**Equation 1: The Z-factor formula**

σp represents the standard deviation of the positive controls and σn is the standard deviation of negative controls. Also, µp represents the mean of the positive controls and µn represents the mean of the negative controls [37].

# CHAPTER FOUR: RESULTS

## Antimalarial Activity

The antimalarial activity of APQ was characterized through Malaria SYBR Green-I Fluorescence assay. This method measures the fluorescence concentration, which correlates to the amount of living parasites in culture. SYBR Green-I is a double-stranded DNA intercalating agent that emits light-induced fluorescence when bound to DNA. The reasoning behind this method is that the *P. falciparum* host, the RBC, does not have DNA of its own as it does not have a nucleus. Hence, the only DNA present in the parasitized RBC is uniquely plasmodial DNA. This allows us to correlate the percent parasitemia to the fluorescence reading. This experiment allows us to measure parasite proliferation after 72-hours of exposure to APQ at different concentrations, in order to generate a dose response curve and an $IC_{50}$ value.

Following MMV parameters, a validated hit should have a submicromolar $IC_{50}$, ideally $\leq$ 500 nM. We determined that APQ has an $IC_{50}$ of 500 nM, successfully complying with such parameter. We also generated a dose-response curve, which is displayed below (Figure 5).

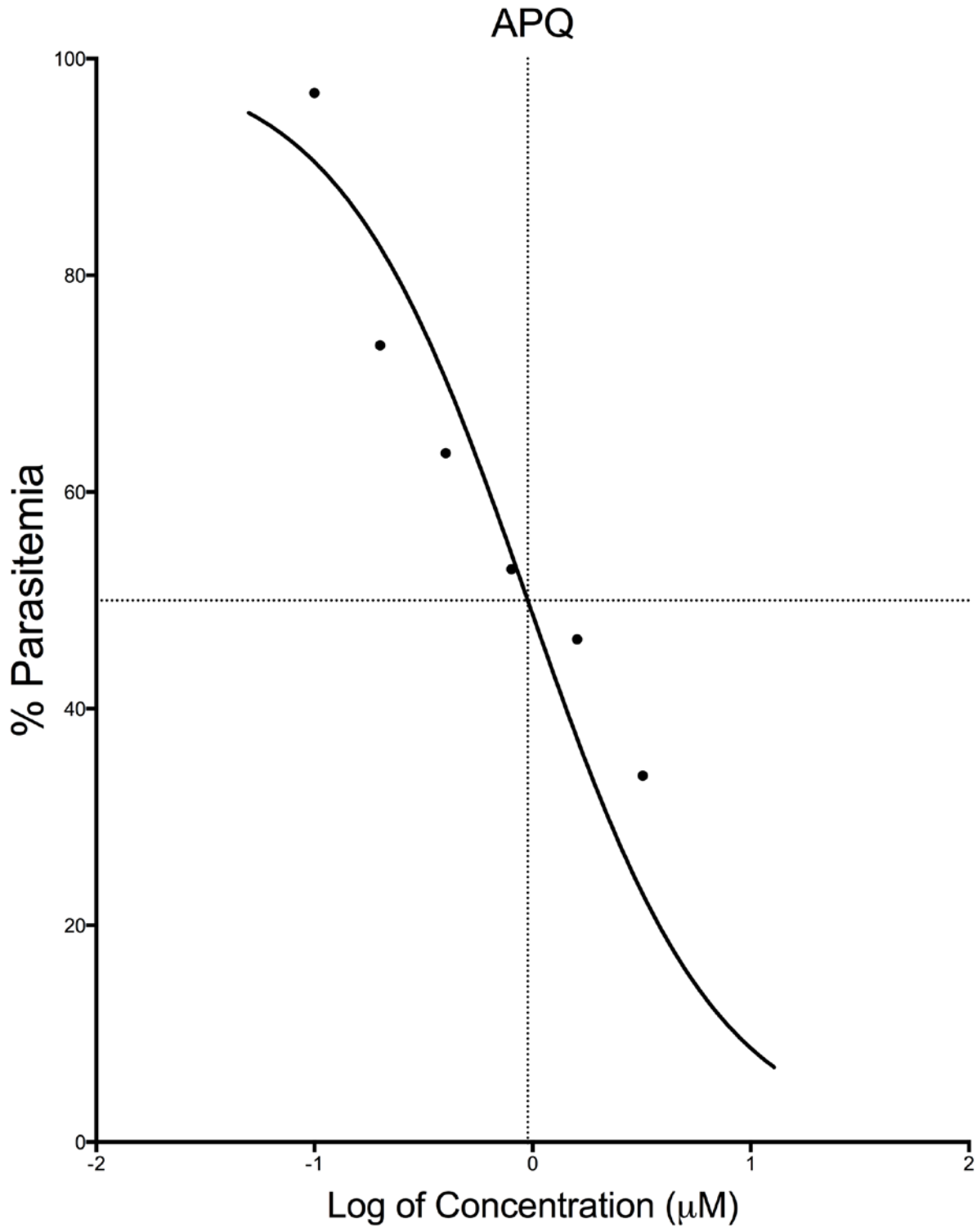



**Figure 5: APQ Half maximal inhibitory concentration ($IC_{50}$) dose response curve against *Plasmodium falciparum* strain Dd2. The $IC_{50}$ value was found at a concentration of 0.5 μM.**

**Cytotoxicity Against Human and Rodent Cell lines**

Dose Response Curve Against NIH3T3 Rodent Fibroblasts

We determined APQ's cytotoxicity to NIH3T3 Rodent Fibroblasts by using the MTS assay. In this assay fibroblasts are incubated in the presence of different concentrations of APQ. MTS is added after the incubation period. MTS is a clear substance that is metabolized in the fibroblast mitochondria into a red-purple dye called formazan. The higher the metabolic rate and the shift in color of the substance correlates to the amount of living cells. This is a colorimetric assay read through absorbance. With the absorbance values, we determined the $IC_{50}$ of our drug in fibroblasts cells to be 175 μM. Comparing this value to the submicromolar $IC_{50}$ of APQ, we can confirm that APQ is up to 350 more potent in *P. falciparum* than in NIH3T3 fibroblasts. Giving us a selectivity index of 350-fold. The MMV criteria for the selectivity parameter in a validated hit is of at least > 10-fold. Our compound, APQ, clearly complies with this criterion. We also generated a dose-response curve, which is displayed below (Figure 6).

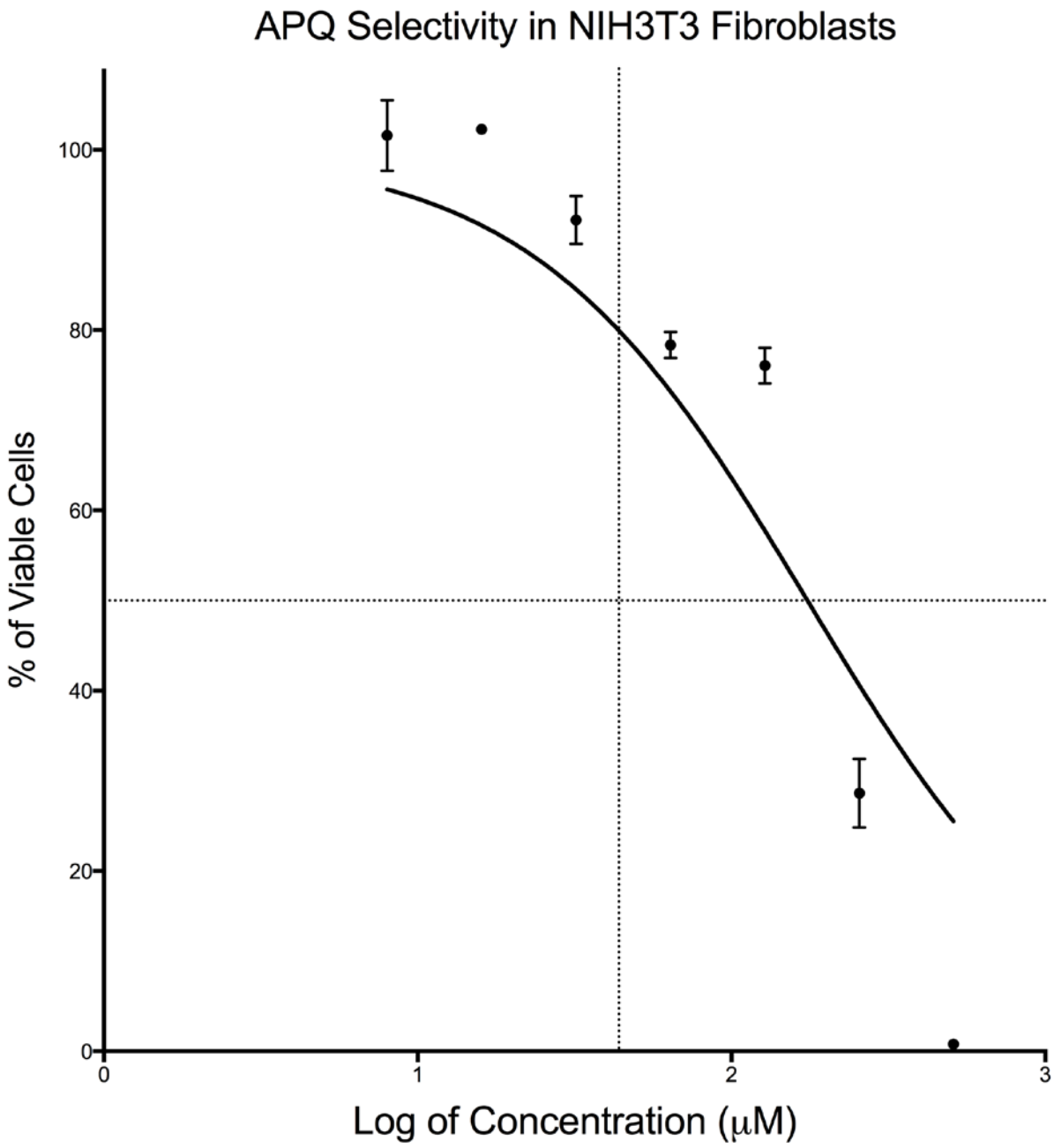



**Figure 6: APQ Half maximal inhibitory concentration ($IC_{50}$) dose response curve against NIH3T3 Fibroblasts. The $IC_{50}$ value was found at a concentration of 175 μM.**

Dose Response Curve Against HepG2 Hepatocytes

The same assay done with NIH3T3 fibroblasts was performed in HepG2 human hepatocytes. Following the same analysis, we determined the $IC_{50}$ of APQ in HepG2 cells to be at 125 μM, meaning 250-fold selectivity for *P. falciparum* and also clearly complying with MMV criteria for selectivity. We also provide a dose-response curve for this assay, which is found below (Figure 7).

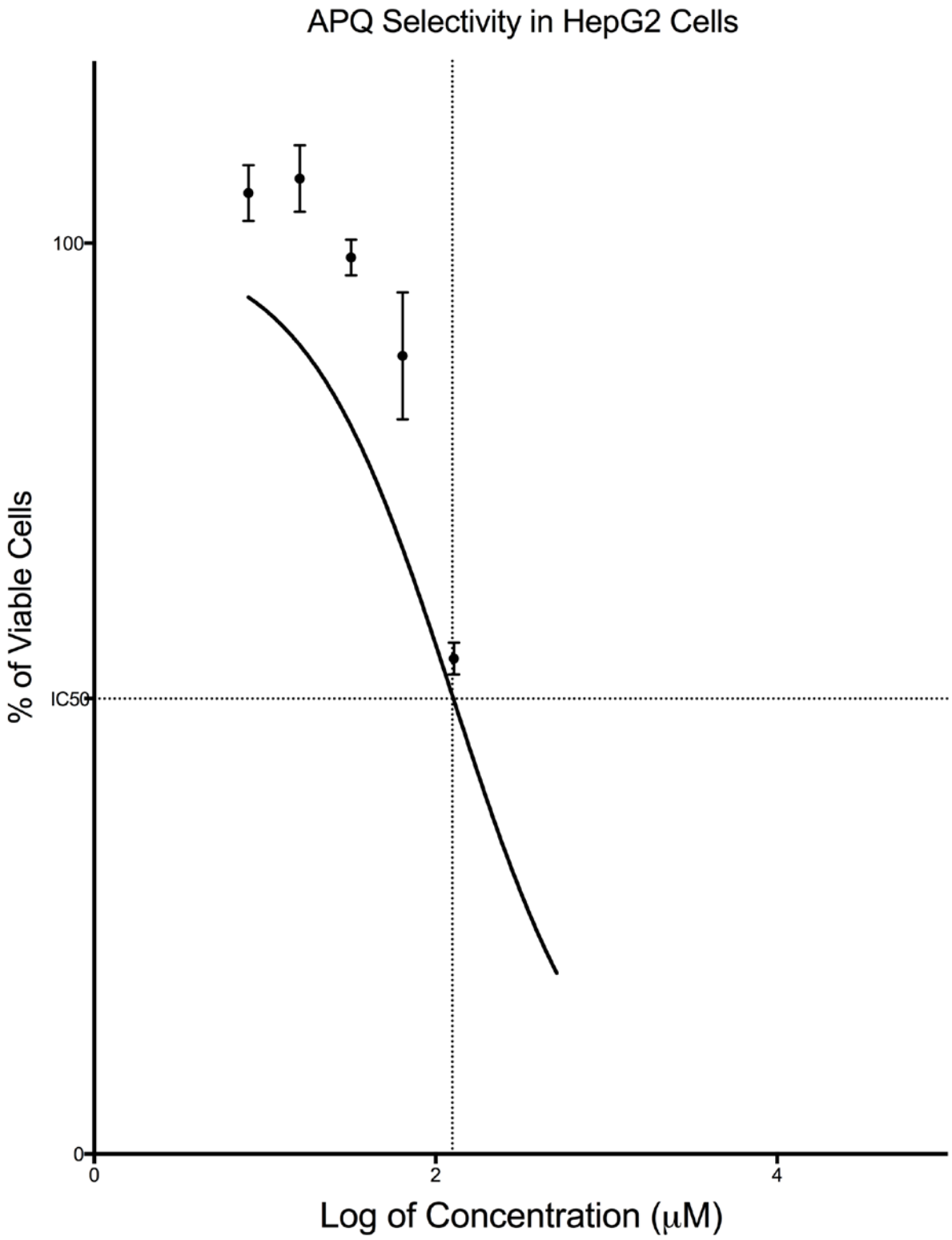



**Figure 7: APQ Half maximal inhibitory concentration ($IC_{50}$) dose response curve against HepG2 Hepatocytes. The $IC_{50}$ value was found at a concentration of 125 μM.**

## Cellular Mechanism of Action

Time Point Assays for Cellular Mechanism of Action Elucidation:

The time point assay was done to determine at which stage of the intraerythrocytic cycle is our drug carrying out its effects. We applied APQ to a synchronized culture at different stages of the blood cycle. These stages were ring (0 h), late ring/early trophozoite (12 h), trophozoite (24 h), schizont (36 h) and finally, reinfection which is represented by a new line of parasites at the ring stage (48 h). We took images at each of these time points in order to qualitatively analyze any changes in the growth pattern of the parasite when in the presence of APQ. We used DMSO as our negative control and Artemisinin as our positive control. DMSO is known to be harmless to the parasites and Artemisinin is in current use as an antimalarial compound. The figure below (Figure 8) shows our findings.

DMSO permits for proper growth, development, reproduction and reinfection of the parasite. Artemisinin arrests growth and development at the trophozoite stage. We determined that APQ also arrests growth and stage progression at the tropozoite stage, it does so however, at a later timepoint than Artemisinin, as its seen by the differences in size and morphology when comparing images of the APQ and Artemisinin effects in the same timepoints.

It is suggested by MMV criteria for compound progression that the cellular mechanism of action should be determined for validated hits in order to progress to lead stages of drug development.

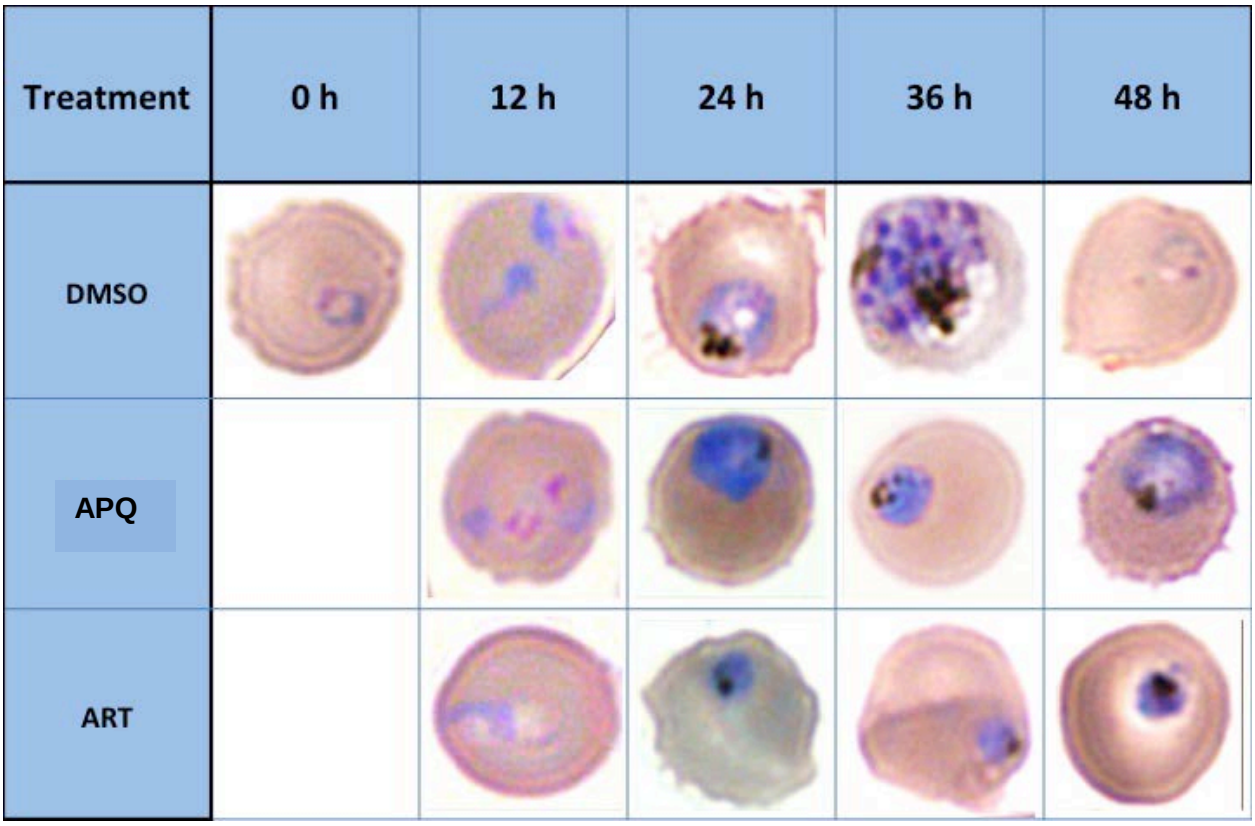



**Figure 8: Varying the life stage at which parasites are exposed to APQ demonstrates that APQ is efficient at arresting parasite development during the trophozoite stage. Timepoint images were taken after exposing the pRBCs to APQ at 5X $IC_{50}$ at 0, 12, 24, 36, and 48 hours post-infection respectively. DMSO and Artemisinin controls were included.**

**Parasitocidal Vs. Parasitostatic Activity**

We determined that our compound, APQ, is parasitocidal after 96-hours of exposure. To determine this, we cultured our parasites in the presence of APQ at 3X and 30X the $IC_{50}$ concentrations. We exposed the parasites to APQ for 24 h, 48h, 72 h, 96 h, and 168 h in simultaneous cultures. We removed APQ from the media after the stated times by repeatedly washing our culture and plating them in fresh media and red blood cells. We then monitored parasite growth through microscopy for up to 300 hours after treatment removal. We found that APQ at a 30X $IC_{50}$ is parasitocidal for all time-points and requires 96-h exposure to eliminate parasitemia completely. DMSO and Artemisinin controls were used.

MMV criteria for validated hits suggest that the parasitocidal vs. parasitostatic information is determined before the compound can be promoted to lead stages. We include graphs of our parasitemia behavior after the drug was removed for the 24 h, 48 h, 72 h and 96 h exposure to APQ below (Figure 9).

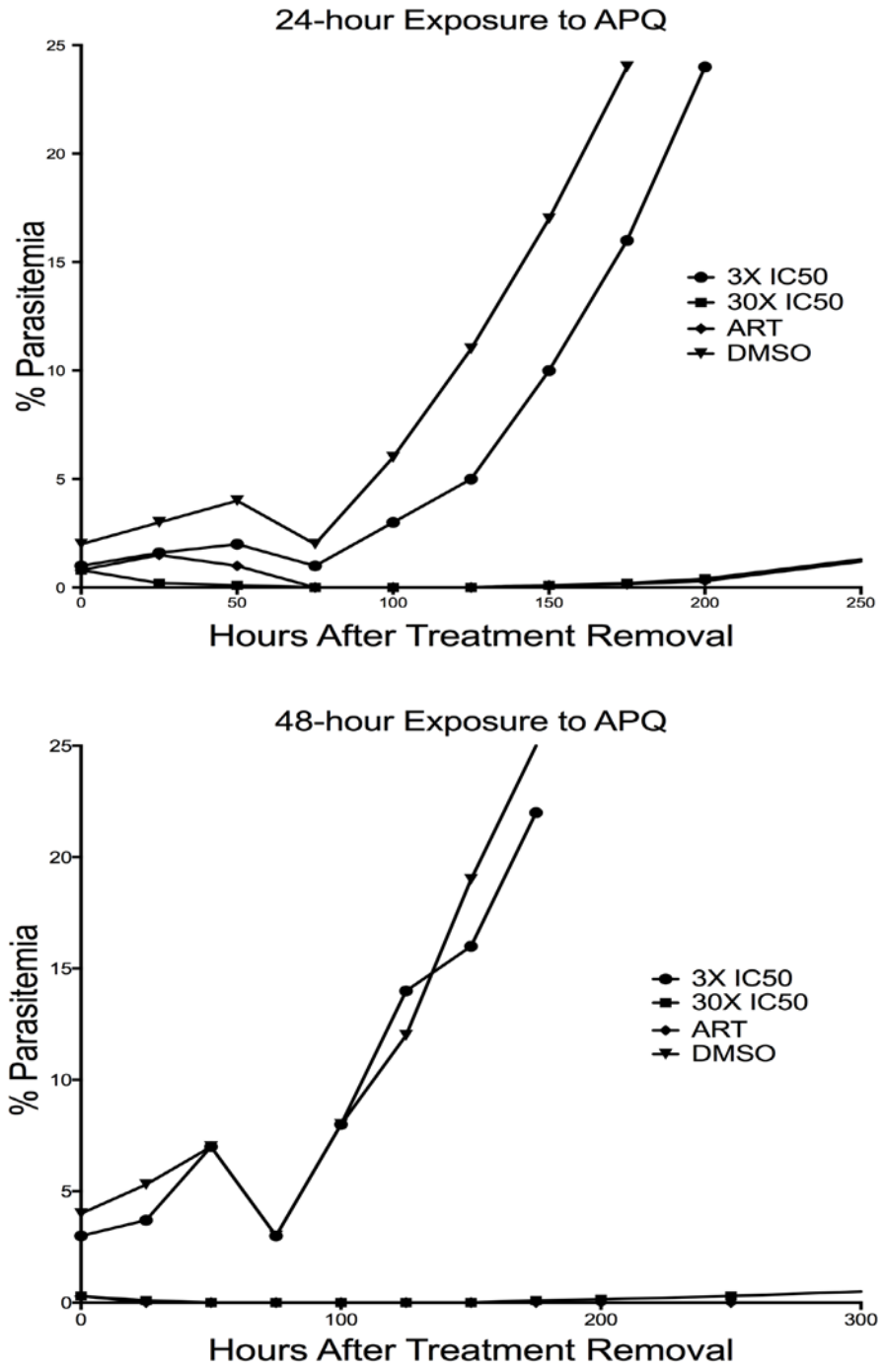



**Figure 9a: Parasitocidal vs. Parasitostatic activity at 24 and 48 hours of exposure to APQ at 3X and 30X $IC_{50}$ concentration**

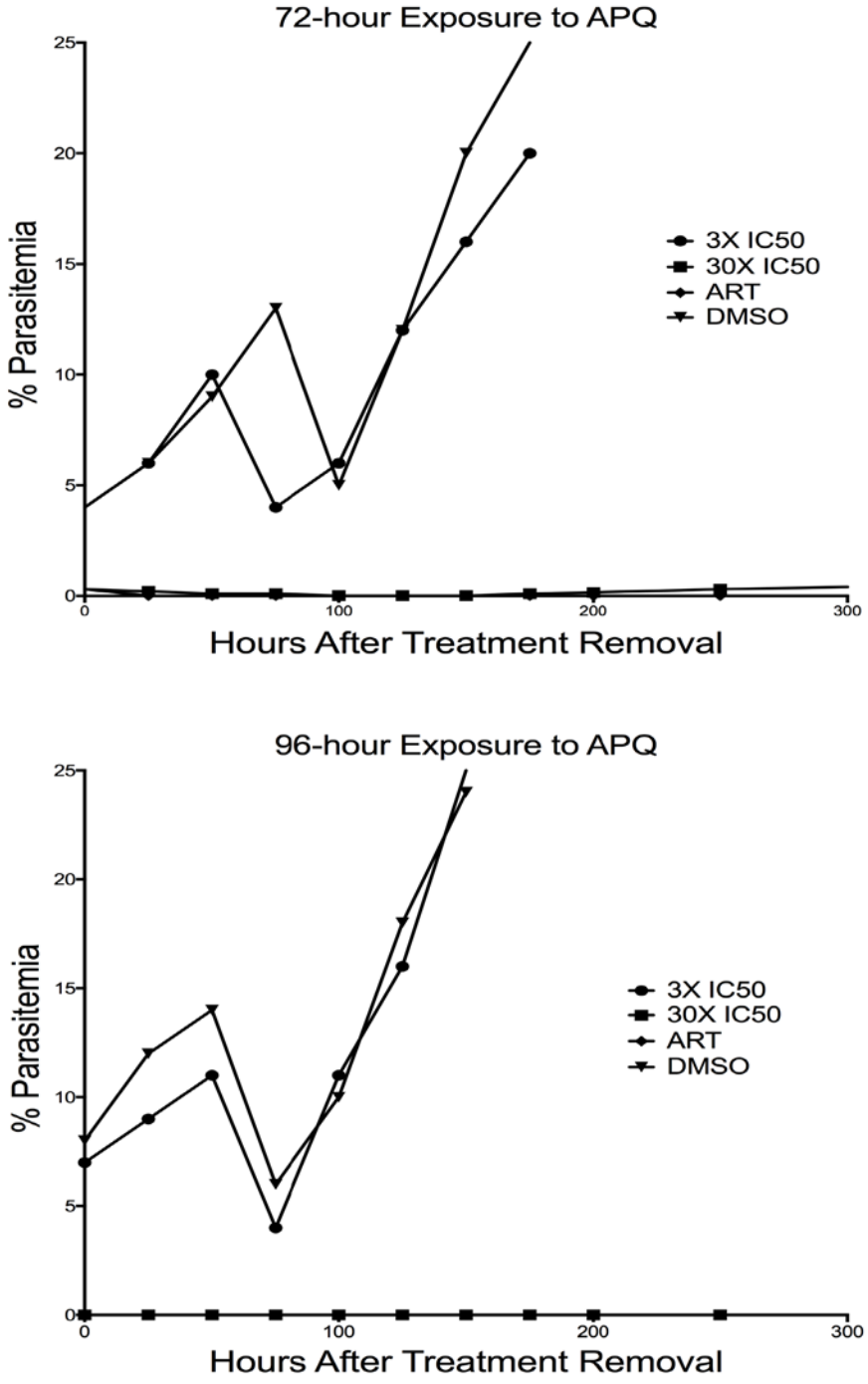
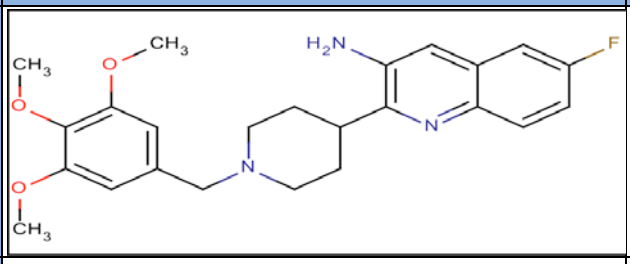
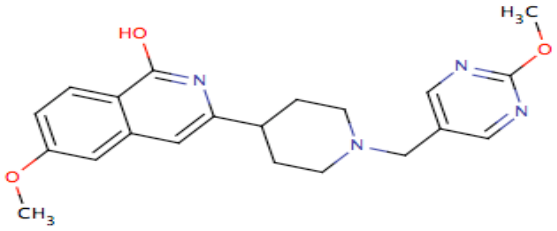
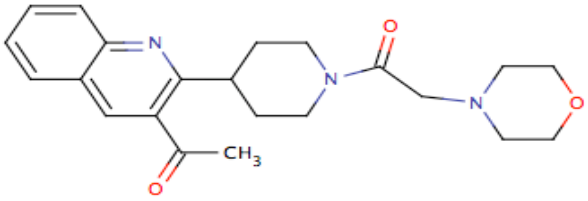
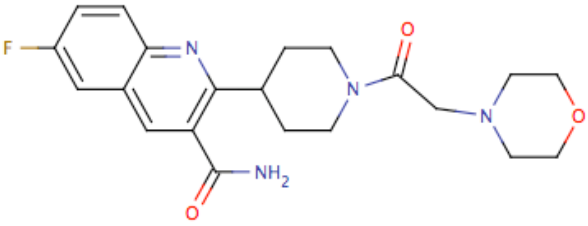
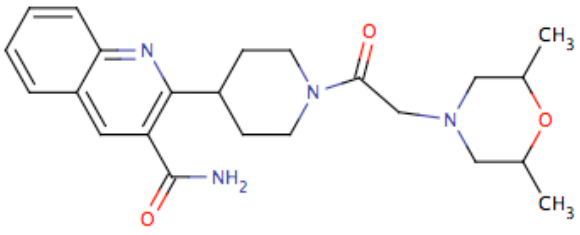



**Figure 9b: Parasitocidal vs. Parasitostatic activity at 72 and 96 hours of exposure to APQ at 3X and 30X $IC_{50}$ concentration. Varying the length of exposure of APQ demonstrates that 96 h exposure of 30X $IC_{50}$ APQ kills *P. falciparum*. *P. falciparum* Dd2 strain in RBC cultures were exposed to 3X $IC_{50}$ or 1.5 μM, 30X $IC_{50}$ or 15 μM of APQ for varying lengths of time before washing the parasites free of drug and reculturing to test if the parasites are viable. DMSO and Artemisinin controls were included.**

**Structure-Activity Relationship**

The structure activity relationship analysis was done by catalogue. We purchased a set of APQ analogs from the same chemical library supplier as our hit-compound (Asinex). We then analyzed their antimalarial efficacy by determining their respective $IC_{50}$ and then their selectivity in NIH3T3 rodent fibroblasts. By analyzing the shift in antimalarial efficacy for the different substituent groups in these analogs, we found that the amino group on position-3 of the quinoline scaffold can be substituted by either an amide or a ketone while maintaining a submicromolar $IC_{50}$, we also determined that the presence of the fluorine on position-7 of the quinoline scaffold also maintains submicromolar $IC_{50}$. On the piperidine scaffold, changing the trimethoxybenzene ring for a morpholine ring also maintains good antimalarial efficacy. The best analog, UCF406, has a combination of a 3-amide and a 7-fluoride on the quinoline scaffold with a 2,6-dimethylmorpholine on the piperidine scaffold, showing an $IC_{50}$ of 0.63 μM. The selectivity is > 100-fold for all the analogs. This structure activity analysis, although limited, since it is not done through medicinal chemistry, gives us a brief idea of what works and what doesn't, analyses with higher scrutiny will be done in the future. Below, we show a table with the name, structure, acitivity and selectivity of each structural analog of our hit-compound APQ (Table 2).

| Compound | Structure | Dd2 IC50 (μM) | 3T3 IC50 (μM) |
|---|---|---|---|
| **APQ** |  | 0.453 | >100 |
| **UCF402** |  | 0.876 | >100 |
| **UCF403** |  | 0.731 | >100 |
| **UCF404** |  | 1.167 | >100 |
| **UCF405** |  | 1.245 | >100 |

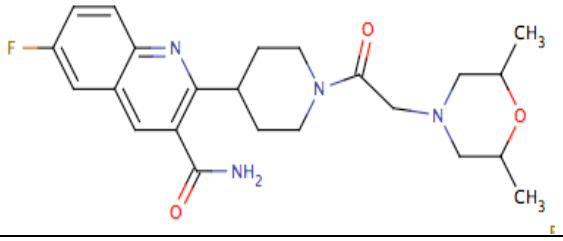
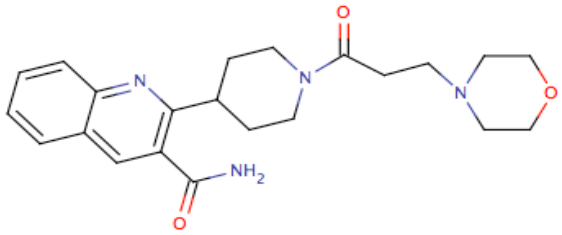
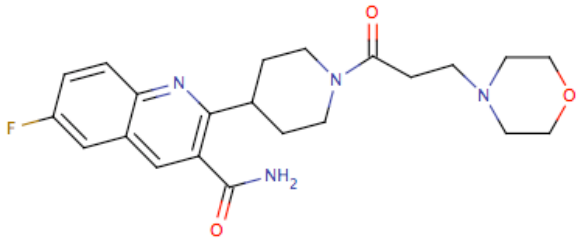
| | | | |
|---|---|---|---|
| **UCF406** |  | 0.6332 | >100 |
| **UCF407** |  | 0.9034 | >100 |
| **UCF408** |  | 0.68725 | >100 |

**Table 2: Structure-Activity Relationship for APQ and its seven structural analogs by demonstrating antimalarial efficacy in Dd2 strain and cytotoxicity in NIH3T3 Fibroblasts.**

**Lipinski Parameters and ADME profile for APQ**

The Lipinski analysis was done through computer software (ChemDraw) and confirmed through chemical database PubChem. The ADME profile was acquired through fee-for-service at the Sanford Burnham Pharmacology Core Facility at Lake Nona, Orlando. Our compound is compliant with Lipinski parameters and has a reasonable ADME profile. We show the values obtained for these parameters in the table below (Table 3).

| Compound APQ | | Obtained |
|---|---|---|
| **Lipinski Parameters (*In Silico*)** | Distribution coefficient - clogP | 2.251 |
| | Molecular Weight | 425.5 |
| | Hydrogen Bond Donors | 2 |
| | Nitrogen and Oxygen Atoms | 6 |
| | Polar Surface Area (sq. Angstroms) | 69.84 |
| **ADME profile** | Solubility pH 7.4 (μg/ml) | >126 |
| | Permeability pH 7.4 (-logPe) | < 2.7 |
| | Microsome Stability (% remaining at 60 min) | 61.34 |
| | Plasma Protein Binding (% bound after 4 h 1 μM/10 μM) | 90.58/79.42 |

**Table 3: Lipinski Parameters and ADME profile for APQ. Values were acquired from the Sanford Burnham Pharmacology Core Facility at Lake Nona, Orlando. Solubility was calculated by kinetic assays, permeability was calculated by parallel artificial membrane permeability assay (PAMPA), Microsome stability was calculated by rate of compound disappearance using CYP450 enzymes in mouse liver microsomes and plasma protein binding was calculated through rapid equilibrium dialysis (RED).**

**MMV Compound Progression Parameters applied to APQ**

The following table (Table 4) summarizes all of our results and compares them with the validated hit compound progression criteria set forth by the MMV in the parameters pertinent to physicochemical properties, pharmacokinetic properties and biological activity. This table allows us to verify and conclude that our compound, APQ, is compliant with all the MMV criteria for validated hit compound progression and is eligible to be promoted as a lead stage compound.

| Parameters | MMV Criteria | APQ Results |
|---|---|---|
| Lipinski Profile | | |
| Distribution coefficient - clogP | < 5 | 2.251 |
| Molecular Weight | < 500 | 425.5 |
| Hydrogen Bond Donors | < 5 | 2 |
| Nitrogen and Oxygen Atoms | < 10 | 6 |
| Polar Surface Area (sq. Angstroms) | < 140 | 69.84 |
| ADME Profile | | |
| Solubility pH 7.4 (μg/ml) | ≥ 10 | >126 |
| Permeability pH 7.4 (-logPe) | Must be known | < 2.7 |
| Microsome Stability (% remaining at 60 min) | Must be known | 61.34 |
| Plasma Protein Binding (% bound after 4 h at 1 μM/10 μM) | Must be known | 90.58/79.42 |
| Biological Activity | | |
| $IC_{50}$ against *P. falciparum* | ≤ 500 nm | 500 nm |
| Selectivity against Human Cell lines (HepG2 must be one of them) | > 10 fold | >100 fold |
| Cellular Mechanism of Action | Must be known | Arrested development at Trophozoite Stage |
| Cidal vs. Static Activity | Must be determined | 100% parasitocidal after 96 hour exposure to 30X $IC_{50}$ |
| Structure-Activity Relationship Analysis | Must be performed | Performed |

**Table 4: Comparison chart between the MMV Validated Hits Progression Criteria for novel compounds [30] and the physicochemical, pharmacokinetic, and biological properties of APQ. Our compound proves to be a promising candidate for the lead stages of drug development, due to its compliance to the parameters established by the MMV.**

# CHAPTER FIVE: DISCUSSION

UCF401 or 3-amino-2-piperidine-quinoline (APQ) has excellent qualities and a huge potential to be developed as a future antimalarial drug. It is compliant with the parameters corresponding to its current development stage (validated hit) set forth by the MMV, the leading organization involved in antimalarial drug discovery and implementation.

APQ has potent efficacy against chloroquine resistant *Plasmodium falciparum* strain Dd2 with $IC_{50}$ values in the submicromolar range. Its efficacy was also previously tested by our laboratory against chloroquine sensitive strain 3D7 showing very similar $IC_{50}$ values, which suggests that its mechanism of action and molecular target differs from chloroquine. APQ was also tested for selectivity against the human cell lines HepG2 hepatocytes and NIH3T3 fibroblasts showing more than 100-fold selectivity for its target. These values promote APQ from a screening hit to a validated hit.

To continue the development progression of our compound, we elucidated the cellular mechanism of action of APQ through cellular timepoint studies. We determined that APQ interacts with essential biological targets necessary for trophozoite stage progression onto the schizont and following developmental stages, since the parasite's intraerythrocytic life cycle is arrested at the trophozoite stage (Figure 8). The next experiment performed on our compound tested for the optimal time of exposure to APQ necessary to kill the entirety of the parasites in culture. This was done through cidal vs. static assays. We determined that exposure to APQ for the duration of 96 hours at a concentration of 30X $IC_{50}$ was sufficient for a complete parasitocidal effect (Figure 9). To corroborate such result, the culture was monitored daily for up to seven days, parasitemia remained at 0% after the 96 hours of exposure as mentioned above.

Following the progression scheme for our compound we analyzed the structure-activity relationships (SAR) by acquiring seven analogs from the Asinex library of APQ compounds. These compounds are close variations of APQ, differing in substituents and functional groups while maintaining the original scaffold. We analyzed these analogs for $IC_{50}$ values against chloroquine resistant *P. falciparum* strain Dd2 and NIH3T3 fibroblasts. All analogs obtained excellent selectivity for *P. falciparum* but their efficacy against the parasites varied. Although the APQ analogs in this set of compounds contain several variations and different combinations of substituents, making difficult to correlate the changes in efficacy to a single difference from APQ, a trend in these modifications can be observed. The substitution of the amino group in position 3 of the quinoline portion by an amide or a ketone also shows submicromolar $IC_{50}$ values. Also, the presence of the fluoride on position 7 of the same compound shows permanency of submicromolar efficacy. Changes like lengthening the alkyl chain on the piperidine, adding methyl groups or the presence of three anisol groups on the benzene ring attached to the piperidine portion contributes to the hydrophobicity of the molecule and this alters the specificity to the yet to be known molecular target. Finally, to comply with the MMV requirements for compound progression, our lab in conjunction with the Sanford Burnham Pharmacology Core Facility at Lake Nona, Orlando, elucidated the pharmacokinetic and physicochemical properties of APQ. Our findings indicate that APQ is in compliance with Lipinski's rule of five and displays a reasonable ADME profile. These features strengthen the potential of APQ.

After performing the pertinent research on APQ for the corresponding stage of drug development, we can argue that APQ has a formidable and well-established incentive to be

further researched and the potential to be promoted to lead and developmental stages of drug development. We believe so because thus far, APQ has successfully complied with all the MMV compound progression parameters established for validated hits, as can be seen in table 4.

**Conclusion**

Malaria is a problem that concerns up to 3.3 billion people that reside in at-risk areas; it infects up to 200 million people yearly and it killed approximately 600 thousand in 2012 alone, as reported by the WHO. These numbers do not take into account the suspected high number of cases that go unreported due to the socioeconomic situation in the geographical regions where malaria is highly prominent. Most of these deaths occur in children under five years of age.

There are current treatments available to cure malaria and mitigate the symptoms caused by it, but the complexity of *P. falciparum,* together with overuse of such treatments and patient incompliance have resulted in the emergence of treatment resistant parasite strains, rendering chloroquine, the former leading form of treatment, almost useless in most areas. Artemisinin combined therapies (ACT) are also beginning to fail due to the same cause in areas such as Southeast Asia [38].

The need for novel therapeutics is top priority to wage war against this disease and eradicate it from our human populations as it has been done in the developed world. The Medicines for Malaria Venture (MMV) has placed a significant effort in this priority, facilitating guidelines and development schemes for scientists in the antimalarial drug discovery field all over the world to come up with such novel therapeutics.

APQ, a nature inspired synthetic product is a validated hit compound with a very strong potential to continue on in the development pipeline. It shows high efficacy and potency against

*P. falciparum* and a very broad selectivity for its target, displaying very low cytotoxicity levels. It acts on the trophozoite stage of the parasite's intraerythrocytic life cycle arresting further development from this stage, and it is efficient at killing the entirety of the parasites in culture following a 96-hour exposure. It is also within the ideal ranges of Lipinski parameters and displays reasonable pharmacokinetic properties. APQ, as a validated hit, complies with all the criteria set forth by the MMV, rendering it as a strong candidate for promotion and progression onto the lead stages of drug development. The APQ chemical scaffold is inspired by natural antimalarials, however it contains a combination of innovative chemical features that had not been tested in the past. With this compound and continued research we plan to make a substantial contribution to the field of antimalarial drug discovery with the ultimate goal of eradicating malaria from humanity.

**Future Directions**

Continued research on APQ must be performed in order to further characterize it and elucidate its properties as a developable drug. This research must of course follow lead stages parameters and criteria set forth by the MMV. Some of these procedures entail *in vivo* studies related to compound activity and pharmacokinetic stability on mouse models, the determination of the rate of resistance development by the parasites, the elucidation of a molecular target and mechanism of action, the possibility of lead optimization using medicinal chemistry and the verification of chemoprophylactic properties by the drug. Finally, it would be also prudent to assess if the compound APQ benefits synergistically by testing changes in its effectiveness when used with combination treatments. These, among other corresponding investigations that must be

done enlighten the direction that should be taken in order to keep advancing this natural like-synthetic compound.

# REFERENCES


1. Centers for Disease Control and Prevention. (2012). **Malaria**. Retrieved from http://www.cdc.gov/Malaria.

2. Chavatte JM, Chiron F, Chabaud A, Landau I. **Probable speciations by "host-vector 'fidelity'": 14 species of Plasmodium from magpies**. *Parasite*. 2007 Mar;14(1):21-37. French. PubMed PMID: 17432055.

3. Gouagna LC, Mulder B, Noubissi E, Tchuinkam T, Verhave JP, Boudin C**. The early sporogonic cycle of Plasmodium falciparum in laboratory-infected Anopheles gambiae: an estimation of parasite efficacy**. *Trop Med Int Health*. 1998 Jan;3(1):21-8. PubMed PMID: 9484964.

4. Cowman AF, Berry D, Baum J. **The cellular and molecular basis for malaria parasite invasion of the human red blood cell**. *J Cell Biol*. 2012 Sep17;198(6):961-71. doi: 10.1083/jcb.201206112. Review. PubMed PMID: 22986493.

5. **The Life Cycle of The Malaria Parasite** [http://www.mmv.org/malaria-medicines/parasite-lifecycle]

6. Rosenthal PJ, Meshnick SR. **Hemoglobin catabolism and iron utilization by malaria parasites**. *Mol Biochem Parasitol.* 1996 Dec 20;83(2):131-9. Review. PubMed PMID: 9027746.

*7.* World Health Organization: **Indoor Residual Spraying: Use of indoor residual spraying for scaling up global malaria control and elimination**. *Global Malaria Programme.* 2006.

8. Jaenson TG, Gomes MJ, Barreto dos Santos RC, Petrarca V, Fortini D, Evora J, Crato J: **Control of endophagic Anopheles mosquitoes and human malaria in Guinea Bissau, West Africa by permethrin-treated bed nets**. *Trans R Soc Trop Med Hyg*. 1994 Nov-Dec;88(6):620–624.

9. Delves M, Plouffe D, Scheurer C, Meister S, Wittlin S, Winzeler EA, Sinden RE, Leroy D: **The Activities of Current Antimalarial Drugs on the Life Cycle Stages of Plasmodium: A Comparative Study with Human and Rodent Parasites.** *PLoS Med* 2012, 9.

10. Wellems TE, Plowe CV: **Chloroquine-resistant malaria**. *J Infect Dis*. 2001 Sep 15;184(6):770-6. PubMed PMID: 11517439.

11. Hyde JE: **Exploring the folate pathway in Plasmodium falciparum**. *Acta Trop*. 2005 Jun;94(3):191-206. PubMed PMID: 15845349.

12. Jeffrey D. Chulay, William M. Watkins, and David G. Sixsmith: **Synergistic Antimalarial Activity of Pyrimethamine and Sulfadoxine against Plasmodium falciparum In Vitro**. *Am J Trop Med Hyg*. May 1984 33:325-330.

13. Ric N. Price: **Potential of Artemisinin-Based Combination Therapies to Block Malaria Transmission.** *J Infect Dis.* (2013) 207 (11): 1627-1629. doi: 10.1093/infdis/jit079.

14. Fidock DA. Drug discovery: **Priming the antimalarial pipeline**. *Nature*. 2010 May 20;465(7296):297-8. doi: 10.1038/465297a. PubMed PMID: 20485420.

15. WHO: **WHO World Malaria Report 2012.** 2012.

16. Kuo-Hsiung Lee**. Discovery and Development of Natural Product-Derived Chemotherapeutic Agents Based on a Medicinal Chemistry Approach.** *Journal of Natural Products* 2010 *73* (3), 500-516.

17. Newman, D. J., Cragg, G. M., and Snader, K. M. **Natural Products As Sources Of New Drugs Over The Period 1981 – 2002**. *Journal of Natural Products* 2013 66, 1022-1037.

18. Guo ZR. **[Modification of natural products for drug discovery]** Yao Xue Xue Bao. 2012 Feb;47(2):144-57. Review. Chinese. PubMed PMID: 22512023.

19. Murugesan D, Kaiser M, White KL, Norval S, Riley J, Wyatt PG, Charman SA, Read KD, Yeates C, Gilbert IH. **Structure-activity relationship studies of pyrrolone antimalarial agents**. *ChemMedChem*. 2013 Sep;8(9):1537-44. doi:10.1002/cmdc.201300177. Epub 2013 Aug 5. PubMed PMID: 23918316.

20. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. **Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings**. *Adv. Drug Deliv. Rev.* 2001. **46** (1-3): 3–26. doi:10.1016/S0169-409X(00)00129-0. PMID 11259830

21. Palm, K., Stenberg, P., Luthman, K., Artursson, P. **Polar molecular surface properties predict the intestinal absorption of drugs in humans**. *Pharm. Res.* **1997**, *14*, 568–571. doi:10.1023/A:1012188625088

22. Terhi Lehtinen Ari Tolonen, Miia Turpeinen, Jouko Uusitalo, Jouni Vuorinen, Risto Lammintausta, Olavi Pelkonen, Mika Scheinin. **Effects of Cytochrome P450 Inhibitors and Inducers on the Metabolism and Pharmacokinetics of Ospemifene.** *Biopharmaceutics & Drug Disposition* (2013), 34: 387-395.

23. Olavi Pelkonen, Miia Turpeinen, Hannu Raunio. **In vivo - in vitro - in silico pharmacokinetic modelling in drug development: current status and future directions.** *Clinical Pharmacokinetics*, 50 (2011) 483 - 491. Review.

24. Jochem Alsenz , Manfred Kansy. **High throughput solubility measurement in drug discovery and development.** *Advanced Drug Delivery Reviews* 59 (2007) 546–567

25. **Drug Distribution to Tissues** [http://www.merckmanuals.com/professional/clinical_pharmacology/pharmacokinetics/drug_distribution_to_tissues.html]

26. Bernard Testa, P. Crivori, M. Reist, PA Carrupt. **The influence of lipophilicity on the pharmacokinetic behavior of drugs: Concepts and examples.** *Perspectives in Drug Discovery and Design.* 2000; 19(1):179-211.

27. Bermejo, M. et al. **PAMPA – a drug absorption *in vitro* model 7. Comparing rat in situ, Caco-2, and PAMPA permeability of fluoroquinolones**. J *Pharm. Sci*., 21: 429-441.

28. LH. Cohen. **Plasma Protein-Binding Methods in Drug Discovery.** *Methods in Pharmacology and Toxicology.* 2004. doi 10.1385/1-59259-800-5:111

29. Waters NJ, Jones R, Williams G, Sohal B. **Validation of a rapid equilibrium dialysis approach for the measurement of plasma protein binding.** *J Pharm Sci.* 2008 Oct;97(10):4586-95. doi: 10.1002/jps.21317. PubMed PMID: 18300299.

30. **Compound Progression Criteria** [www.mmv.org/sites/default/.../Compound_progression_criteria.pdf]

31. Brooks WH, Guida WC, Daniel KG. **The significance of chirality in drug design and development.** *Curr Top Med Chem*. 2011;11(7):760-70. Review. PubMed PMID:21291399.

32. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, (193):673-675.

33. Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, Waters NC. **Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening**. *Antimicrob Agents Chemother*. 2007 Jun;51(6):1926-33. PubMed PMID: 17371812.

34. Lambros C, Vanderberg JP: **Synchronization of Plasmodium falciparum erythrocytic stages in culture**. *J Parasitol*. 1979 Jun;65(3):418-20. PubMed PMID: 383936.

35. Nallan L, Bauer KD, Bendale P, Rivas K, Yokoyama K, Hornéy CP, Pendyala PR, Floyd D, Lombardo LJ, Williams DK, Hamilton A, Sebti S, Windsor WT, Weber PC, Buckner FS, Chakrabarti D, Gelb MH, Van Voorhis WC**. Protein farnesyltransferase inhibitors exhibit potent antimalarial activity**. *J Med Chem*. 2005 Jun2;48(11):3704-13. PubMed PMID: 15916422.

36. Mosmann T: **Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays**. *J Immunol Methods*. 1983 Dec 16;65(1-2):55-63. PubMed PMID: 6606682

37. Zhang JH, Chung TD, Oldenburg KR: **A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays**. *J Biomol Screen*. 1999;4(2):67-73. PubMed PMID: 10838414.

38. Fidock DA, Rosenthal PJ, Croft SL, Brun R, Nwaka S: **Antimalarial drug discovery: efficacy models for compound screening**. *Nat Rev Drug Discov*. 2004 Jun;3(6):509-20. Review. PubMed PMID: 15173840.